AWARD NUMBER: W81XWH-14-1-0207

TITLE: A Novel EphA4-Based Small Molecule-Based Therapeutic Strategy for Prevention and Treatment of Post-Traumatic Osteoarthritis

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REPORT DATE: September 2016

TYPE OF REPORT: Revised Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2016	Revised Annual Report	15Aug2015 - 14Aug2016

4. TITLE AND SUBTITLE
A Novel EphA4-Based Small Molecule-Based Therapeutic Strategy for Prevention and Treatment of Post-Traumatic Osteoarthritis

5a. CONTRACT NUMBER
W81XWH-14-1-0207

5b. GRANT NUMBER
W81XWH-14-1-0207

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Post-traumatic osteoarthritis (PTOA) is a painful and debilitating disease of the joints, characterized by intra-articular inflammation, deterioration of articular cartilage, and degenerative changes to peri-articular and subchondral bone. There is currently no effective therapy. This project capitalizes our discovery that the forward signaling of EphA4 receptor has both an anti-catabolic effect (on osteoclasts/chondroclasts) and an anabolic effect (on chondrocytes) on skeletal tissues and seeks to develop a small molecule-based therapy for PTOA that involves merely direct injection of a soluble EphA4-acting EfnA-fc protein into the injured synovium. We will first confirm that EfnA-fc-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating neutrophils/monocytes would reduce survival and the release of pro-inflammatory cytokines and MMPs, but, in articular chondrocytes, would enhance chondrogenesis *in vitro*. We will then optimize a small molecule EfnA-fc-based strategy involving injections of an EphA4-binding EfnA-fc into the joint by determining an appropriate EfnA-fc ligand for EphA4, an optimal dosage, and the time course of the effect, and an appropriate duration between injections. Finally, we will determine whether the small molecule EfnA-fc-based therapeutic strategy can prevent PTOA-mediated degradation of articular cartilage in early PTOA, and also can promote regeneration of articular cartilage in established PTOA. If this therapy is effective, it will improve the quality of life of PTOA patients, not only in the active duty military personnel and the veteran population, but also in the civilian population. It may even allow the warrior to return to active duty. This proposal has high military benefits.

15. SUBJECT TERMS
Osteoarthritis; post-traumatic osteoarthritis; synoviocytes; articular cartilage regeneration; intra-articular fractures; chondrocytes; articular chondrocytes; articular cartilage degradation.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	UU	33	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Post-traumatic osteoarthritis (PTOA) is characterized by intra-articular inflammation, deterioration of articular cartilage, and degenerative changes to peri-articular and subchondral bone. This project capitalizes our discovery that the EphA4 forward signaling has an anti-catabolic effect on osteoclasts/chondroclasts but an anabolic effect on chondrocytes on skeletal tissues and seeks to develop a small molecule-based therapy for PTOA that involves merely direct injection of a soluble EphA4-acting EfnA-fc protein into the injured synovium. This therapy on the one hand would activate the forward signaling of EphA4 in infiltrating monocytes and in osteoclasts/chondroclasts to suppress the release of pro-inflammatory cytokines and proteases to degrade articular cartilage, and on the other hand it acts on articular chondrocytes to promote regeneration of the damaged articular cartilage. This project first confirms that EfnA-fc-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating neutrophils/monocytes would reduce survival and the release of pro-inflammatory cytokines and MMPs, but, in articular chondrocytes, would enhance chondrogenesis *in vitro*. We will develop a mouse intra-articular fracture model of PTOA for use in this study. We will then optimize a small molecule EfnA-fc-based strategy involving injections of an EphA4-binding EfnA-fc into the joint by determining an appropriate EfnA-fc ligand for EphA4, an optimal dosage, and the time course of the effect, and an appropriate duration between injections. Finally, we will determine whether the small molecule EfnA-fc-based therapeutic strategy can prevent PTOA-mediated degradation of articular cartilage in early PTOA, and also can promote regeneration of articular cartilage in established PTOA. If this therapy is shown to be effective, it will improve the quality of life of PTOA patients, not only in the active duty military personnel and the veteran population, but also in the civilian population. It may even allow the warrior to return to active duty.

2. KEYWORDS:

Osteoarthritis; post-traumatic osteoarthritis; EphA4; regenerative therapy; synoviocytes; articular cartilage regeneration; intra-articular fractures; chondrocytes; articular chondrocytes; articular cartilage degradation.

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Study hypotheses:

- Hypothesis 1 - activation of EphA4 in synovial monocytes reduces release of pro-inflammatory cytokines and proteinases, but enhances chondrogenesis in articular chondrocytes *in vitro*.
- Hypothesis 2 – the small molecule EfnA-fc-based strategy involving intra-articular injection of an EfnA-fc into PTOA joint of a mouse can be developed and optimized for PTOA.
- Hypothesis 3 - the small molecule EfnA-fc-based therapy is effective in preventing PTOA development and in treating advanced PTOA in mice.

Tasks/Approach:

- Task 1 - To obtain necessary regulatory approval and to recruit appropriate personnel prior to the initiation of the project.
    - Subtask 1.1: To obtain the required regulatory review and approval for use of animal subjects in the research *(completion date: 07-14-2014)*.
    - Subtask 1.2: To recruit an appropriate research technician for the proposed work

(*completion date: 02-15-2016*).

- Task 2 – To determine whether the EfnA4-fc-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating monocytes/macrophages would reduce cell survival and the release of pro-inflammatory cytokines and MMPs, but in articular chondrocytes, would enhance chondrogenesis and their survival in vitro *(completion date: 09-15-2016)*.
    - Subtask 2.1 – To isolate synoviocytes and infiltrating monocytes/macrophages as well as articular chondrocytes from the injured synovium of C57BL/6J mice one week after the intra-articular fracture *(completion date: 02-28-2015)*.
    - Subtask 2.2 – To determine whether EfnA4-fc treatment of monocytes/macrophages and inflamed synovial fibroblasts would reduce release of pro-inflammatory cytokines and MMPs as well as reduces cell survival in vitro *(completion date: 09-15-2016)*.
    - Subtask 2.3 – To determine whether EfnA4-fc treatment of articular chondrocytes would promote chondrocyte proliferation, and maturation as well as reduces cell survival in vitro *(completion date: 09-15-2016)*.

- Task 3 – To develop and optimize a small molecule- and EphA4-based strategy involving injection of an EphA4-binding EfnA-fc chimeric protein into the inflamed joint for PTOA.
    - Subtask 3.1 – To identify an appropriate EfnA-fc chimeric protein for use in the proposed therapy *(~15% completion)*.
    - Subtask 3.2 – To identify an appropriate dosage of EfnA-fc chimeric protein for use in the proposed therapy.
    - Subtask 3.3 – To determine the time course of effects after administration of the EfnA-fc chimeric protein.
    - Subtask 3.4: To compare the relative efficacy after a single administration vs. multiple administrations of EfnA-fc.

- Task 4 – To demonstrate that the optimized small molecule-, EfnA-fc-based therapy can prevent PTOA-induced degradation of articular cartilage during early phase of PTOA and can promote regeneration of articular cartilage in later phase of PTOA.
    - Subtask 4.1 – To determine whether administration of the optimized EfnA4-based therapy to the synovium of the injured joint shortly after the intra-articular tibial plateau fracture would prevent degradation of articular cartilage and development of PTOA (~5% completion).
    - Subtask 4.2 – To determine whether administration of the optimized EfnA4-based therapy to the synovium of the injured joint after PTOA is fully developed would promote regeneration of articular cartilage and cure PTOA.

During the second year of this project (the reporting period), we focused on completion of work of Task 2. We also initiated some of the proposed work of Subtasks 3.1 and 4.1.

What was accomplished under these goals?

Accomplishments made during year 2 (15-09-2015 to 14-09-2016):

1. The major research activities:
   - *To complete Task 2 (Determination of whether the EfnA4-mediated activation of the forward signaling of EphA4 in synoviocytes and infiltrating monocytes/macrophages would reduce cell survival and the release of pro-inflammatory cytokines and MMPs, but in the articular chondrocytes, would enhance chondrogenesis and their survival in vitro).*

During year 1, we have successfully finished all proposed work for subtask 2.1 (Isolation of synoviocytes and articular chondrocytes) and subtask 2.3 (Demonstration that EfnA4-fc enhances activity of articular chondrogyctes). The major activity on Task 2 has, therefore, been aimed to complete the remaining work of subtask 2.2 (Demonstration that EfnA4-fc inhibits activity activity of the inflamed synoviocytes).

- *To begin work of Task 3 (Development and optimization of a small molecule- and EphA4-based strategy involving injection of an EphA4-binding EfnA-fc chimeric protein into the inflamed joint for PTOA).*

During the past year, we also began the proposed work on Subtask 3.1, which was to optimize the EfnA4-fc-based strategy to prevent development of PTOA after an intra-articular tibial plateau injury to the knee joint. Our immediate objective was to compare the relative efficacy of biweekly intra-articular injection of an effective dose of EfnA4-fc on the prevention of the PTOA. This work would need reliable and sensitive quantitative analytical methods to assess the relative severity of the PTOA. However, during our investigations, we found that none of the current analytic methods reported in the literature was reliable, because they are highly subjective and are based primarily on histology of the joint. Bone histology is a good qualitative proof for development of PTOA, but it is not a reliable quantitative method, since it is so subjective to the site of examination, which is highly variable and is also subjective to the various confounding factors, including variations in morphology and bone and cartilage architecture, and the intrinisic variabilities in the various histological staining methods. Because it is essential that we need to have a reliable quantitative method for quantitative comparison of the relative severity of PTOA after each treatment protocol, we have decided to spend some efforts in improving and optimizing the quantitative assessment of the relative severity of PTOA after the closed intra-articular tibial plateau fracture injury. The remaining work of Task 3 (Subtask 2 and 3) will be focus of the next reporting year.

- *To initiate work of Task 4 (To demonstrate that the optimized small molecule-, EfnA-fc-based therapy can prevent PTOA-induced degradation of articular cartilage during early phase of PTOA and can promote regeneration of articular cartilage in later phase of PTOA*).

We have begun testing of the efficacy of the regimen of biweekly injection of an effective dosage of EfnA4-fc into injured knee joints could prevent PTOA development.

2. Specific Technical Objectives:

   a. Subtask 2.2: To determine whether EfnA4-fc treatment of monocytes/macrophages and inflamed synovial fibroblasts would reduce release of pro-inflammatory cytokines and MMPs as well as reduces cell survival in vitro
   b. Subtask 3.1: To develop the EfnA4-fc-based therapy for PTOA – to identify appropriate form and dosages of EfnA-fc protein for use.
   c. Subtask 4.1: To determine whether injection of a high dosage of EfnA4-fc every two weeks into injured knee joints could prevent PTOA development.

3. Detailed Description of Accomplishments – significant results or key outcomes, major positive and negative findings:

a. *Subtask 2.2: To determine whether activation of forward signaling of EphA4 in synovial fibroblasts by EfnA4-fc treatment would inhibit the functional activities of synovial fibroblasts in vitro*.
The synovial membrane, which is the thin inner layer located between the joint capsule and the joint cavity, produces a clear, viscous fluid (synovial fluid) to reduce friction between the joint cartilages during movement. Synovial membrane is divided into two compartments – the outer layer (subintima) and the inner layer (intima). The inner layer is mainly composed of two cell types, synovial macrophages (specialized macrophage cells) and synovial fibroblasts. Synovial fibroblasts are stromal cells of mesenchymal origin that display many characteristics common with fibroblasts, such as expression of several types of collagens and protein vimentin, a part of cytoskeletal filaments. Unlike other fibroblasts, synovial fibroblasts also secrete unique proteins, that are normally absent in other fibroblast lineages. These include lubricin (also known as PRG4, a protein crucial for the joint lubrication), cadherin-11, VCAM-1, various integrins and their receptors, CD55 (a characteristic protein that is often used as a marker protein to identify synovial fibroblasts in the synovium by immunohistochemistry). Synovial fibroblasts represent the main source of hyaluronic acid and also other glycoproteins, major components of the synovial fluid. These extracellular matrix components and secreted factors are essential for the maintenance of the normal environment of the synovial fluid and articular surface of the joint. During joint inflammation, e.g., rheumatoid arthritis, synovial fibroblasts take on an aggressive, invasive phenotype, breaking down cartilage by secreting and activating various matrix metalloproteinases (MMPs), whilst their production of secreted factors such as soluble RANKL promotes osteoclast differentiation, survival and activity, contributing to bone erosion and subchondral bone erosion.

To confirm that the fibroblast-like cells that we isolated from the synovial membrane of the intact and damaged knee joints of C57BL6/J mice were bona fide synovial fibroblasts, total RNA was isolated from the fibroblast-like synoviocytes isolated from synovial membranes of three separate mice and cDNA was generated. We then measured the relative expression levels of three marker genes (S100A4, collagen α1 chain, and α-SMA) and cyclophilin (the housekeeping gene) using the SyBr Green-based qPCR assay. S100A4 gene was chosen as a marker of activated synoviocytes, because S100A4 (a member of the S100 calcium-binding proteins) is expressed at site of invasion in rheumatoid arthritis synovium and modulates production MMPs, and because increased S100A4 protein in circulation and locally at sites of inflammation, particularly at sites of joint destruction, has been linked to the process of aggressive fibroblast behavior contributing to the pathogenesis of chronic autoinflammatory diseases such as rheumatoid arthritis. Col2α1 mRNA level was measured, since it is recognized as one of the known extracellular proteins made by synovial fibroblasts. We also include αSMA (as a negative control) in the analysis since this gene is a known marker for vascularization and not a marker for synovial fibroblasts. Our isolated synovial fibroblasts expressed substantial levels of S100A4 and Col2α1 mRNA (but not αSMA) at the relative expression level of ~10-15% of that of the housekeeping gene, cyclophilin (data not shown). However, there were substantial variations in the measurements of each mRNA. This is probably due to the fact that the amount of RNA isolated from each cell population was very low. Nevertheless, these findings are consistent with our contention that these are bona fide synovial fibroblasts.

As an initial test of the effects of intra-articular tibial fracture on the activation status of synoviocytes in the injured knee joint, the relative levels of S100A4, Col2α1, or αSMA mRNA in the synovial fibroblasts isolated from the injured knee joint of three mice at one week post-fracture were measured and compared with those in synovial fibroblasts isolated from synovial membranes of the contralateral uninjured knee joint. As shown in **Fig. 1**, the expression level of S100A4 in synovial fibroblasts of the injured knee was increased almost 190-fold, when compared to that in synovial fibroblasts of the intact contralateral control

knee joint. Intriguingly, the expression level of αSMA was reduced to ~10% of that in the synoviocytes of the uninjured knees. This huge increase in S100A4 mRNA, which has been associated with the functional activity and inflammation, along with the almost 7-fold increase in the expression in Col2α1 mRNA level, is consistent with the interpretation that the synovial fibroblasts in the injured knee joint were activated.

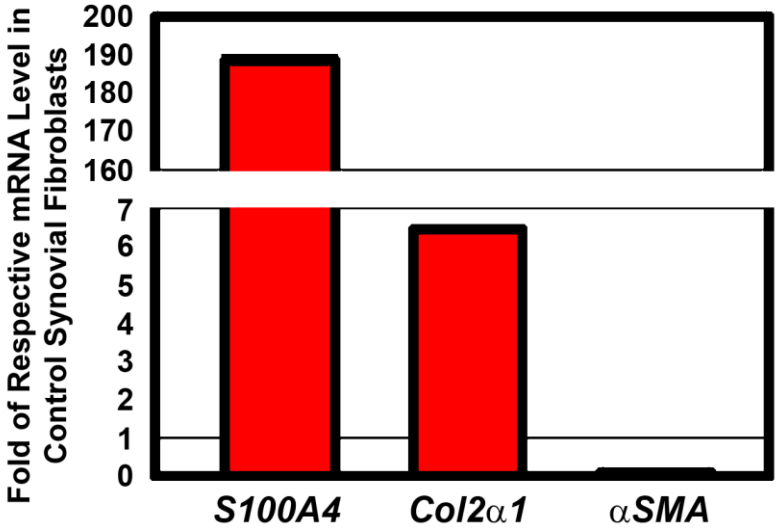

Figure 1. Injury to synovial membranes significantly upregulated expression levels of S100A4 and col2α1, but not αSMA mRNA. Total RNAs were isolated from injured synovial membranes of three C57BL/6J mice 7 days after intra-articular tibial plateau fracture, and cDNAs were generated. The mRNA level of the indicated genes was determined by the SyBr Green-based qPCR. Results are shown as relative percentage of the respective level of the housekeeping gene, cyclophilin. Because the assays showed large variations, respective error bars are not shown.

Synovial fibroblasts are stromal cells of mesenchymal origin, and expansion and development of stromal cells of mesenchymal origin requires a number of growth, stem and differentiation factors. Our initial efforts in culturing and expanding these synoviocytes were largely unsatisfactorily. We consulted literature and several commercial companies and learnt that commercial culture media for human synovial fibroblasts are available. We purchased these media and used these media to expand and grow mouse synovial fibroblasts with very positive results. However, the key ingredients in these media were treated as proprietary trade secrets and we were unable to prepare these special culture media ourselves. In any event, we are now able to isolate, culture, and expand mouse synovial fibroblasts in vitro that allows us to have large amounts of synovial fibroblasts isolated from basal or inflamed synovium for our in vitro experiments.

Once we worked out the method to culture and expand synovial fibroblasts, we performed a qRT-PCR experiment to determine the relative expression profile of the various members of the Eph's and their ligands, ephrin's (Efn's). Briefly, confluent petri dishes of "non-inflamed" synovial fibroblasts at passage 4 were extracted for total RNA using the Lipid Extraction kit. The purity and concentration of RNA was analyzed by the ratio of absorbance at 260 nm to absorbance at 280 nm. Three µg RNA was used for the reverse transcription reaction to produce cDNA. The resulting cDNA was used for measurement of the relative levels of the various Ephs and ephrins by the Sybr-Green-based quantitative PCR. The house keeping gene, cyclophilin, was also measured for comparison. **Table 1** shows the number of critical cycles (Ct) of each Eph or ephrin mRNA (average of three independent measurements). Based on our experience, Ct cycle numbers greater than 33 cycles represent "noise level" of the method, indicating no appreciable expression level of the test mRNA species (shown in red). ΔCt represents the difference in Ct between the

test mRNA species and corresponding housekeeping gene, cyclophilin, which calculated the relative abundance of each test mRNA in term of relative fold of the cyclophilin mRNA level. We should note that the lower the critical cycle the higher the expression level of the given mRNA species.

Table 1. Relative expression level of the various ephrins and Ephs in cultured synoviocytes.

mRNA species	Ct	ΔCt	Fold of cyclophilin
Cyclophilin	23.143	------	(1)
Efn A1	22.795	-0.348	1.272795
Efn A2	24.223	1.080	0.473029
Efn A3	29.827	6.684	0.009726
Efn A4	24.395	1.252	0.419866
Efn A5	25.159	2.016	0.247243
Efn B1	24.148	1.006	0.497925
Efn B2	23.622	0.479	0.717475
Efn B3	22.183	-0.960	1.94531
Efn B4	24.725	1.582	0.334019
Efn B5	22.843	-0.300	1.231144
EphA1	25.211	2.068	0.23849
EphA2	21.233	-1.910	3.758091
EphA3	22.744	-0.399	1.318594
EphA4	29.572	6.429	0.011606
EphA5	27.88	4.737	0.037499
EphB1	34.452	11.309	0.000394
EphB2	22.728	-0.415	1.333299
EphB3	22.413	-0.730	1.658639
EphB4	25.478	2.335	0.198196

With exception of EphB1, these synoviocytes expressed substantial levels of each major member of the Eph family, with EphA2 being the most abundant member. Similarly, these synoviocytes also expressed each of the common members of the ephrins with EfnB3 being the most abundant and EfnA3 the least abundant. The basal EphA4 expression in these cells was substantial, albeit it was lower than some of the other Eph's. For this in vitro work, we arbitrarily used EfnA4-fc as the soluble ligand to activate the EphA4 signaling in these synoviocytes.

Table 2. Basal relative expression levels of cytokines in cultured synovial fibroblasts.

mRNA species	Ct	ΔCt	Fold of cyclophilin
Cyclophilin	24.680		(1)
IL-1α	>45	-----	--------
IL-1β	41.655	16.975	--------
IL-4	>45	-----	--------
IL-6	30.989	6.309	0.012613
IL-10	40.511	15.831	--------
IL-13	36.653	11.973	--------

Our working hypothesis predicts that activation of the EphA4 forward signaling (through treatment with EphA4-fc chimeric protein) in synoviocytes and infiltrated monocytes would suppress their catabolic actions through reduction of production of inflammatory cytokines and/or degradative MMPs. To test this hypothesis in vitro, we first determined their relative expression levels of the various inflammatory

cytokines, such as IL-1α, IL-1β, IL-4, IL-6, and IL-17α (**Table 2**) and the various MMPs (**Table 3**). We included two anti-inflammatory cytokines, IL-13 and IL-10, in **Table 2** for comparison. Results were obtained from a single preparation of the cultured synovial fibroblasts after 4 passages. Thus, statistical analysis of the expression results was not feasible. *Again, Ct cycle numbers greater than 33 cycles, which indicate no appreciable expression level, are shown in red*.

To our surprise, synovial fibroblasts expressed very low basal levels of the test inflammatory cytokines, with only IL-6 was considered to be detectable (**Table 2**). These findings raise the interesting possibility that synoviocytes may not been the cellular source of pro-inflammatory (or anti-inflammatory) cytokines during development of PTOA or OA. Thus, the likely cell source would be the infiltrating monocytes.

Table 3. Basal relative expression levels of MMPs in cultured synovial fibroblasts.

mRNA species	Ct	ΔCt	Fold of cyclophilin
Cyclophilin	24.166		(1)
Mmp3	30.407	6.241	0.013221
Mmp9	22.491	-1.675	3.193194
Mmp13	18.41	-5.756	54.04166

In contrast, synovial fibroblasts expressed very high levels of MMPs, especially Mmp13, which was 54-fold higher than the mRNA level of the housekeeping gene, cyclophilin (**Table 3**). Therefore, the synovial fibroblasts can be a significant cellular source of degradative enzymes.

We next tested whether activation of the EphA4 forward signaling in synovial fibroblasts would alter expression profile of these cytokines and MMPs. Very briefly, after the cultured synoviocytes reached 80% confluence, 10 ng/mL of EfnA4-fc soluble chimeric protein was added to each well of the 6-well plates for 24 hours. Total RNA was isolated, and cDNA was prepared. The relative level of each test cytokine and Mmp mRNA was then measured by Sybr-Green-based qRT-PCR. Results are shown in **Figure 2** as percentage of each corresponding mRNA levels of the vehicle-treated control cell cultures. Because this preliminary experiment was performed in a single preparation of isolated synoviocytes, we did not perform statistical analysis of these results.

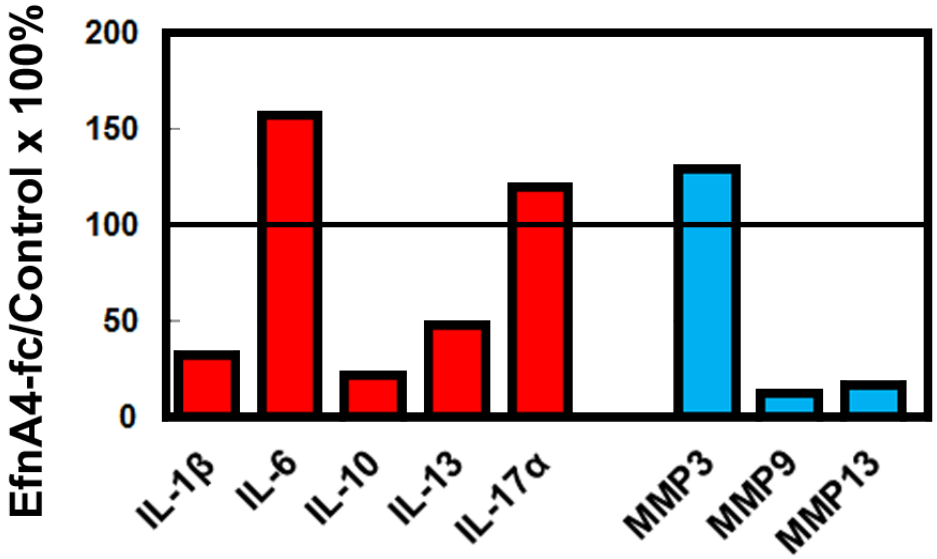

Figure 2. Effects of EfnA4-fc-mediated activation of the EphA4 signaling on mRNA expression levels of inflammatory cytokines (red bars) and MMPs (light blue bars) in cultured synoviocytes. Results are shown as percentage of the corresponding mRNA level of respective vehicle-treated cells. The solid line indicates the corresponding 100% level of respective mRNA of the vehicle-treated controls.

Figure 2 shows that the EfnA4-fc treatment down-regulated several key inflammatory cytokines, including IL-1β, IL-10, IL-13, but not IL-6 or IL-17α. Similarly, the expression level of MMP9 and MMP-13 mRNA (but not the MMP-3 mRNA) was greatly suppressed. This preliminary experiment, however, has several caveats. First, other than IL-6, the expression levels of the test cytokines were very low. Therefore, the observed reductions in the level of these pro-inflammatory cytokines in response to EfnA4-fc might not be reliable and must therefore be confirmed with other means. Second and more importantly, our preliminary studies have indicated that synoviocytes expressed multiple forms of Eph, and many of them can bind EfnA4. Thus, we cannot definitively be certain that the observed effects were exclusively due to activation of the EphA4 forward signaling. In the future, we will perform more definitive studies to confirm that the observed effects were consequence of an activation of the forward signaling of EphA4 in these cells. Nevertheless, these findings are consistent with our hypothesis that activation of the EphA4 forward signaling suppressed the expression of pro-inflammatory cytokines and MMPs in synoviocytes.

To further test our overall hypothesis that the EphA4 forward signaling has anabolic actions in articular chondrocytes but catabolic actions in synoviocytes and infiltrating monocytes, our original strategy was to treat articular chondrocytes and synoviocytes with soluble EphA4-fc chimeric protein to suppress the EphA4 forward signaling and to determine the consequences in the release of pro-inflammatory cytokines and degradative enzymes from synoviocytes and in the maturation and activity of articular chondrocytes. Our rationale was that addition of the soluble EphA4-fc chimeric protein in target cells would serve as decoy receptors (and sequestrate) for all EphA4-binding Efns, rendering the forward signaling of EphA4 in these cells un-stimulated and largely nonfunctional. However, most of our findings with this approach were inconclusive and even conflicting (data not shown). It was probably because as shown in **Table 1**, synoviocytes express substantial amounts of multiple forms of EphA4-binding Efns. Because soluble EphA4-fc chimeric protein not only could suppress the forward signaling of EphA4 but also activates reverse signaling of these EphA4-binding Efns through directly binding to the various cell surface Efns, and the reverse signaling of many of these Efns (e.g., EfnB1, B2, B4, and A2) has both stimulatory and inhibitory effects on the functions and differentiation of both osteoblasts and osteoclasts. Thus, the unintended activation of the reverse signaling of these EphA4-binding Efns on articular chondrocytes and/or synoviocytes would also affect their cell functions. As a result, the interpretation of these studies becomes very challenging and impossible. Consequently, we needed an alternative strategy to further test our hypothesis.

Our current VA Merit Review project involved characterization of the osteoclasts and bone phenotypes in EphA4 null transgenic mice. Once the EphA4 null mice were euthanized and used for isolation of pre-osteoclasts, we believe that the remaining tissues, including the knee joint, of EphA4 null synovium and/or EphA4 null articular chondrocytes could be used in this subtask to determine the consequences of deficient expression of EphA4 in the release of pro-inflammatory cytokines and degradative enzymes from synoviocytes and in the maturation and activity of articular chondrocytes. Our rationale was that addition of the soluble EphA4-fc chimeric protein in target cells would serve as decoy receptors (and trap) for all EphA4-binding Efns, rendering the forward signaling of EphA4 in these cells un-stimulated and largely nonfunctional. Accordingly, we salvaged the knee joints from the carcasses of the euthanized EphA4 null mice and WT littermates, which could be used for isolation of synoviocytes or articular chondrocytes after the carcasses were discarded from the other study. We reasoned that synoviocytes of the EphA4 null synovium would be completely devoid of EphA4 expression, which would be an excellent alternative strategy to test our hypothesis that deficient in EphA4 forward signaling in synoviocytes would lead to enhancement of the catabolic action, exemplified by upregulation of pro-inflammatory cytokine and MMPs expression.

For this work, synovium was isolated from three sets of discarded knee joints of 8-week-old EphA4 null and WT littermates of our VA Merit Review project. After non-synovial tissues were removed and synovium rinsed three times in phosphate-buffered saline, synovial extract was prepared. Total RNA was isolated from each synovial extract, cDNA was prepared, and relative RNA levels of each test gene along with cyclophilin (the housekeeping gene) were determined by qRT-PCR. Results are shown as relative fold of respective WT control, determined by the ΔΔCT method. We first assessed effects of deficient EphA4 expression on cellular functions (as reflected by the relative expression levels of inflammatory cytokines and degradative enzymes) of the synovium tissue. We compared the relative expression levels of several relevant inflammatory cytokines (i.e., IL-1β, IL-6, and TNFα) and degradative enzymes (i.e., Mmp3, Mmp6, and Mmp13) in total RNA isolated from synovium of EphA4 null mice and compared them with relative expression levels of these genes of WT littermates. We also compared the expression levels of two marker genes of articular cartilage, aggrecan (ACAN) and proteoglycan 4 (also known as lubricin) (PRG4) for comparison. The relative RNA levels of each test cytokines and Mmps were determined (and normalized against corresponding cyclophilin mRNA). The results shown as relative fold of each test mRNA in WT synovium (**Figure 3**).

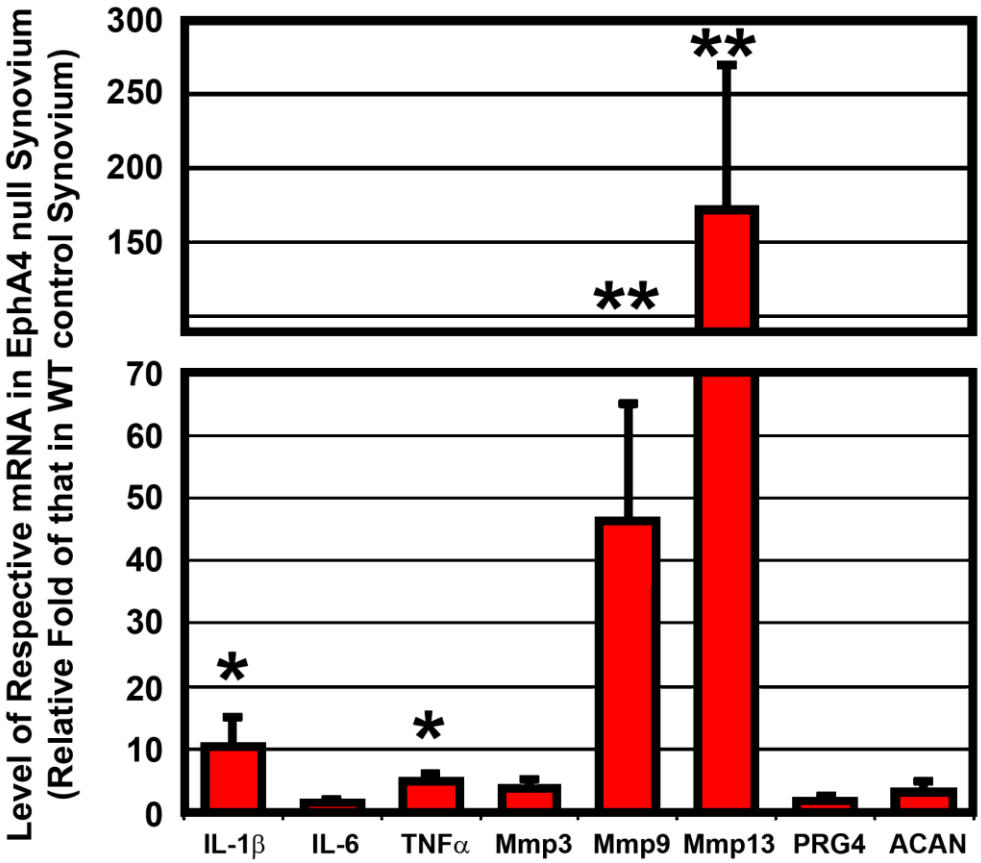

Figure 3. Relative basal mRNA levels of relevant inflammatory cytokines, degradative enzymes and cartilage genes in synovium of EphA4 null knee compared to those in synovium of WT littermate knees. Results are shown as percentage of the corresponding mRNA level in synovium of age- and gender-matched WT littermates (mean ± SEM, n=6-9). *P<0.05; and **P<0.01.

Interestingly, the relative level of IL-1β and TNFα mRNA, two key pro-inflammatory cytokines that are known to play an pathogenic role in PTOA/OA, but not IL-6, was significantly increased by 5- to 10-fold in synovium of EphA4 null mice when compared to those in WT synovium. Similarly, the expression levels of the two major Mmps (Mmp9 and Mmp13) that are known to mediate the degradative actions in PTOA/OA

in EphA4 null synovium were also 45- to 150-fold, respectively, greater than those in WT synovium. Mmp3 has also been implicated to have a role in degradation of articular cartilage in arthritis. Although deficient expression of EphA4 in cells within the synovium also increased its Mmp3 mRNA levels, the increase was not statistically significant. Nevertheless, these findings are consistent with the premise that deficiency in EphA4 signaling in synovium leads to local upregulation of expression of inflammatory cytokines and degradative Mmps. However, deficient expression of EphA4 did not alter the relative expression levels of ACAN and PRG4 in cells of the synovium. We should, however, emphasize that the relative expression level of these two articular cartilage marker genes in the synovium tissue were very low, which are not at all surprising since synovium is not the major site of articular cartilage production.

Our previous studies (reported in the last year annual report) have demonstrated that treatment of isolated articular chondrocytes with soluble EphA4-fc chimeric protein significantly suppressed the proliferation and differentiation (reflected by expression of marker genes of differentiated articular chondrocytes), which are consistent with our interpretation that suppression of the EphA4 forward signaling could lead to suppression of articular chondrocytes proliferation and maturation. However, as indicated above, the soluble EphA4-fc chimeric protein approach has significant limitations. Accordingly, we sought to confirm this conclusion by evaluating the consequence of deficient EphA4 expression in EphA4 null articular cartilage on its functional activity. Our focus was on the comparison of the relative expression levels of the various marker genes of articular cartilage, such as aggrecan (ACAN), lubricin (PRG4), type 2 collagen (Col II), type 10 collagen (Col X), and Sox 9, in the isolated articular cartilage layer of three EphA4 null mice with those of three WT littermates (Figure 4). Deficient expression of EphA4 in the articular cartilage layer significantly reduced the relative expression levels of ACAN, Col II, Cox X, and Sox 9 by 70% to 90%. Intriguingly, the relative expression level of PRG4, which has been used as a tissue-specific marker for articular cartilage, was not significantly different in EphA4 articular cartilage layers from that in WT articular cartilage.

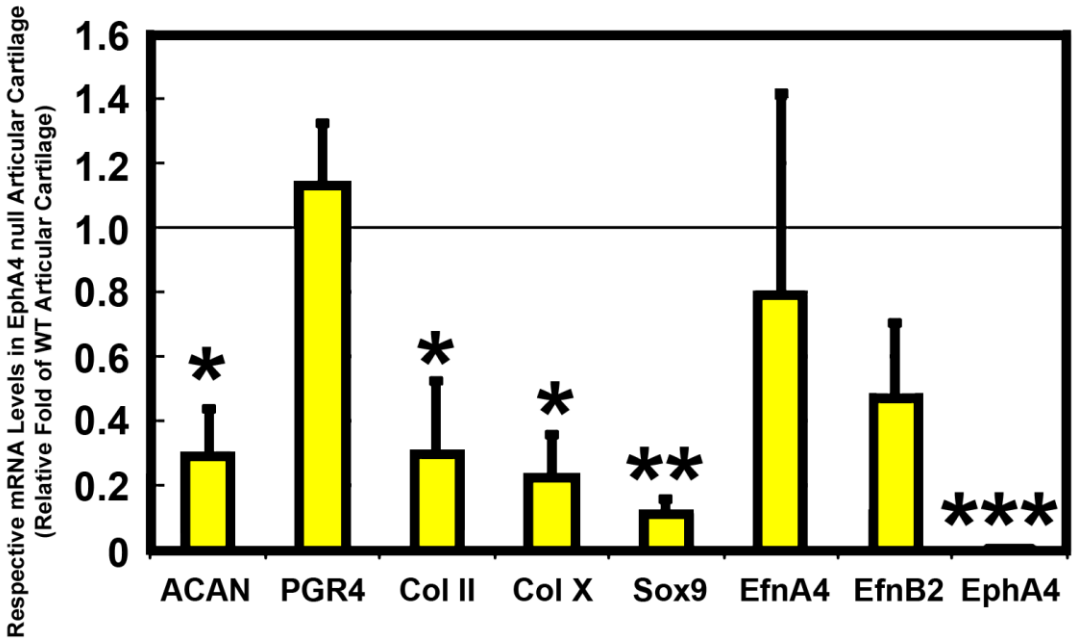

Figure 4. Effects of deficient EphA4 expression on relative basal mRNA levels of articular cartilage marker genes, EphA4, EfnA4, and EfnB2, in the articular cartilage layers. Results are shown as percentage of the corresponding mRNA level in EphA4 null articular cartilage layers of age- and gender-matched WT littermates (mean ± SEM, n=3 per group). *P<0.05; **P<0.01, and ***P<0.001.

The significance of the lack of an apparent effect of deficient EphA4 expression on the PRG4 mRNA level in articular cartilage is unclear at this time. It raises the possibility that PRG4 biosynthesis in articular cartilage might not be subjected to regulation of the forward signaling of EphA4. Nonetheless, these findings are entirely compatible with our concept that the EphA4 forward signaling plays an anabolic role in the differentiation and maturation of articular chondrocytes and in the biosynthesis of articular cartilage.

One of the caveats of using tissues from EphA4 null mice is that deficient EphA4 expression could also reduce the reverse signaling of the various EphA4-binding Efns. Hence, our studies with EphA4 null cells did not rule out the remote possibility that the observed changes in respective EphA4 null tissues were consequence of reduction of the reverse signaling of the Efns rather than deficient in EphA4 forward signaling. As an initial means to address this alternative possibility, we measured the relative expression levels of EphA4 and two major EphA4-binding Efns, namely, EfnA4 and EfnB2. We reasoned that if the observed effects of deficient EphA4 expression were due to suppression of the reverse signaling of the EphA4-binding Efns, we expect that there should be significant feedback upregulation of the expression level of the EphA4-binding Efns. **Figure 4** confirms that the articular cartilage tissue derived from EphA4 null mice was indeed devoid of EphA4 expression, as there was no detectable EphA4 mRNA in the EphA4 null articular cartilage layers. Importantly, deficient expression of EphA4 did not increase (if anything, there were decreases) mRNA levels of EfnA4 and EfnB2 in EphA4 null articular cartilage layers. Based on this piece of circumstantial evidence, we tentatively conclude that the observed effects in EphA4 null tissues were probably due to suppression of the forward signaling of EphA4. Our future studies will confirm this possibility with more sophisticated approaches.

We would like to make an observation regarding EphA4 null mice. Accordingly, our original plan was to test the hypothesis that EphA4 signaling plays an important determining role in development or prevention of PTOA/OA in vivo was to assess the effects of injection of EphA4-fc chimeric protein in the injured joint before or after development of PTOA to determine our concept that suppression of the EphA4 signaling would worsen PTOA development. However, in light of our in vitro studies, it seems likely that this approach would not be productive. Consequently, we plan to submit an amendment to our local IACUC and ACURO for approval to use EphA4 null mice. Our revised plan would then determine if PTOA development would be exacerbated in EphA4 null mice when compared to age- and sex-matched wild-type littermates, after approval for the amended studies are approved by our local IACUC and ACURO.

In summary, Task 2 is now 100% completed.

b. *Subtask 3.1: To develop the EfnA4-fc-based therapy for PTOA – to identify appropriate form and dosages of EfnA-fc protein for use*.

Task 3 is to develop and optimize an EfnA-fc-based therapy for PTOA/OA. We use the mouse closed intra-articular tibial plateau model as the animal model for PTOA. Because our objective is to determine if the local intra-articular injection of Efn-fc chimeric protein would quantitatively improve on the severity of PTOA, it is critically important that we have a reliable quantitative method to measure the relative severity of PTOA. Accordingly, we divide this Subtask into four distinct phases. The first phase is to assess the reliability of and validate the currently used assessment methods for PTOA, which is needed for evaluating relative efficacy of the treatment. In the second phase, we will work out the most appropriate delivery method for administration of the EfnA-fc chimeric protein into the inflamed knee joint. This is a very important criterion needed for the successful application of our proposed therapeutic strategy. The third phase then focuses on identification of the most effective soluble ligand of EphA4 by comparing the relative efficacy of the various forms of EfnA-fc. The final phase would then be the dose-dependent study of the selected soluble EfnA-fc. The reporting period has been focusing on the first two phases. The last two phases will be the aims for the next reporting year.

There is currently no established and reliable quantitative means to assess the severity of PTOA/OA. Most experimental studies relied on qualitative assessments of destruction of articular cartilage and subchondral bone to determine the extent of PTOA/OA. The three most current quantitative methods are based on grading systems for clinical symptoms of OA/PTOA. They are: 1) the histopathologic assessment-based macroscopic evaluation grading system devised by Collins (i.e., Collins' score) in 1940's; 2) a microscopic histologic histochemical grading system (HHGS) developed in 1971 by Markin et al., (Minkin's OA score); and 3) the OsteoArthritis Research Society International (OARSI) scorin system, which was developed in 1998 by an OA working group of OARSI to devise a "standard" OA grading system based on current pathophysiologic knowledge. The Collins system graded OA severity as Grades I-IV using extensive qualitative descriptions of cartilage surface texture, lesion size, and bony changes. Mankin's histopathology grading system is based on microscopic evaluation of decalcified sections of surgically removed OA femoral heads stained by Safranin O with Light Green counterstain. This system used a 14-point score based on a composite cellular changes, histochemical presence of Safranin O matrix staining and architectural changes (erosion, vessel penetration tidemark). The OARSI Osteoarthritis Cartilage Histopathology Assessment System is based on histologic features of OA progression. It divides OA into 7 grades: Grade 0 to Grade 6, and five stages: Stage 0 to Stage 4. All the grades and stages assume that the tissue reaction observed has microscopic features characteristic of OA activity. Grade is defined as OA depth progression into articular cartilage, and is an index of the severity or biologic progression of the OA process. This assumes that OA involvement of deeper cartilage is a more advanced disease and a good indicator of progressive disease. Stage is defined as the horizontal extent of articular cartilage involvement within one side of a joint compartment irrespective of the underlying grade. Stage 0 has no OA activity seen, while stage 1 to 5 represents, <10%, 10-25%, 25-50%, and >50% OA activity, respectively. The semi-quantitative OA score is defined as assessment of combined OA grade (OA severity) and OA stage (OA extent): Score = grade x stage.

For our work, we initially sought to develop a similar histology-based disease scoring system to the Minkin's OA scoring system. To achieve that, we need to first characterize the histologic characteristics of PTOA developed in our intra-articular tibial plateau fracture mouse model. The detailed procedure for the mouse closed intra-articular tibial plateau fracture method has been reported in previous progress report.

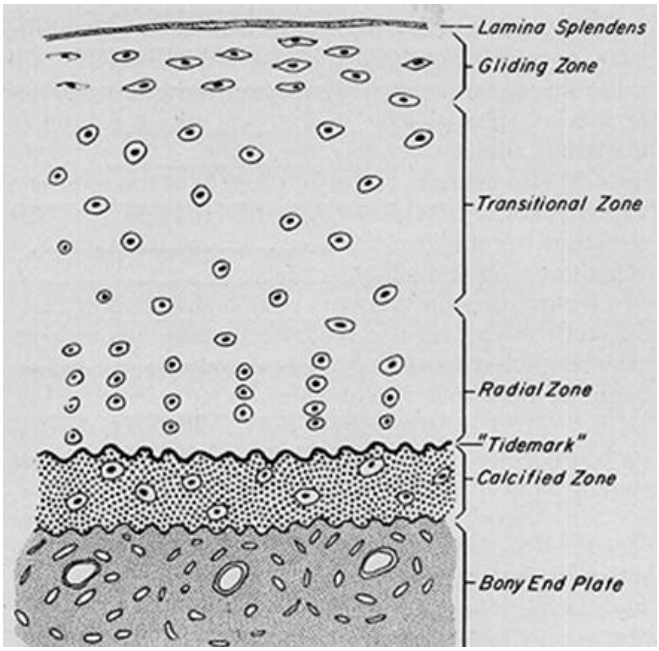

Figure 5. A schematic representation of the longitudinal view of the morphology of articular cartilage.

Figure 5 represents a schematic view of the longitudinal section of the articular cartilage. The articular cartilage is generally divided into 4 characteristically distinct zones: 1) Zone 1 (also known as gliding, tangential, or superficial zone). This is the thinnest zone, composed of flattened ellipsoid chondrocytes that lie parallel to the joint surface and are covered by a thin film of synovial fluid, called "lamina splenens or lubricin. Parallel arrangement of the fibrils are responsible for providing the greatest tensile and shear strength. Disruption of this zone alters the mechanical properties of the articular cartilage and thus contributes to the development of osteoarthritis. This layer also acts as a filter for the large macromolecules, thereby protecting the cartilage from synovial tissue immune system. 2) Zone 2 (also known as transitional or intermediate zone). The cell density in this zone is lower, with predominantly spheroid-shaped chondrocytes, embedded in abundant extracellular matrix. The large diameter collagen fibers are randomly arranged in this zone. 3) Zone 3 (also known as radial or deep zone), which is the broadest zone. This zone contains the largest diameter of collagen fibrils and highest concentration of proteoglycans. Chondrocytes in this zone are arranged perpendicular to the surface and are spheroidal in shape, but the cell density is lowest among all zones. 4) Zone 4 (Calcified cartilage zone). This is a thin zone with substantial calcification that separates the Radial zone from the subchondral bone. The cells here are smaller and fewer but arranged in rows. In some areas the cells are totally buried in individual Calcified Sepulchers, suggesting that that they may have a very low level of metabolic activity. The junction between the radial and calcified zones is distinctly visible as a basophilic line in stained preparations of articular cartilage. This faint but distinct bluish line is known as the "Tidemark". Subchondral bone lies immediately underneath the calcified zone. Injury to the joint could cause significant damages to all four zones, resulting in destruction of the articular cartilage and subchondral bone, leading to development of PTOA. The extent of damages to these zones is frequently used as criteria for the severity of PTOA/OA.

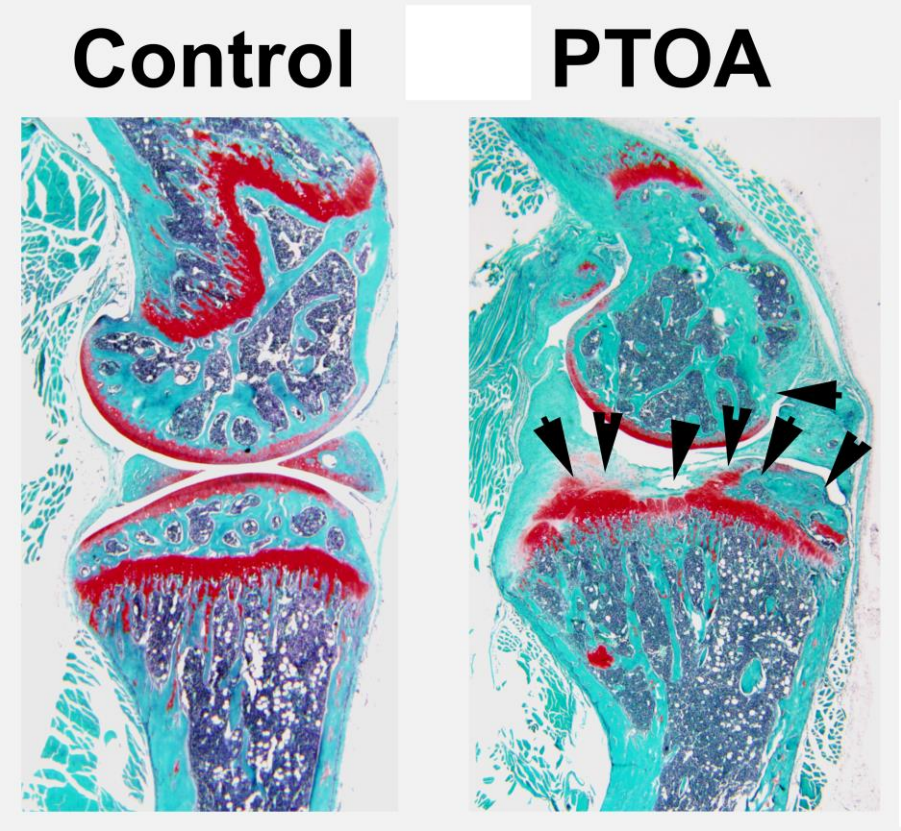

Figure 6. Comparison of the Safranin-orange/fast green-stained articular cartilage in the injured joint (right panel) versus the intact contra-lateral joint (left panel) five weeks post-surgery. The eroded (degraded) articular cartilage (Arrowheads) = thinner layers of articular cartilage with less staining intensity.

Accordingly, we performed Safrain-O staining of proteoglycan of articular cartilage (stained red), as an assessment of degrees of damage of some of these zones, on longitudinal thin sections of injured right and intact contralateral knee joints at 5 weeks post-fracture at the site of ~1/3 from the lateral side of the knee joint. **Figure 6** clearly demonstrated that there were significant amounts of erosion of the articular cartilage of the injured right (PTOA) joint at both the tibial and femoral surfaces, when compared to the uninjured contralateral left joint. These findings clearly indicate the development of PTOA in the injured right knee at as early as 4-5 weeks post-fracture.

To further investigate the effects of PTOA development on the four zones of articular cartilage, a longitudinal section at the tibial articular cartilage at 125 µ laterally from the medial edge of the knee joint of the PTOA and control contralateral joint was stained for safarin-orange and examined at 40 x magnification (**Figure 7**). Both zones 1 and 2 of the articular cartilage were destroyed and appeared to be eroded and were not readily identifiable on the PTOA joint. In comparison with the control joint, Zone 4 of the PTOA articular cartilage became largely disorganized. To investigate this further, we histologically stained a serial slide for alcian blue counterstained with fast red (**Figure 8**), which confirmed destruction of zone 1 and 2 and disorganization of zone 3 and 4. It also confirms an increase in remodeling of subchondral bone as reflected by the highly irregular shape of the underneath subchondral bones. Consequently, PTOA indeed caused destruction of all zones of the articular cartilage as well as in subchondral bone.

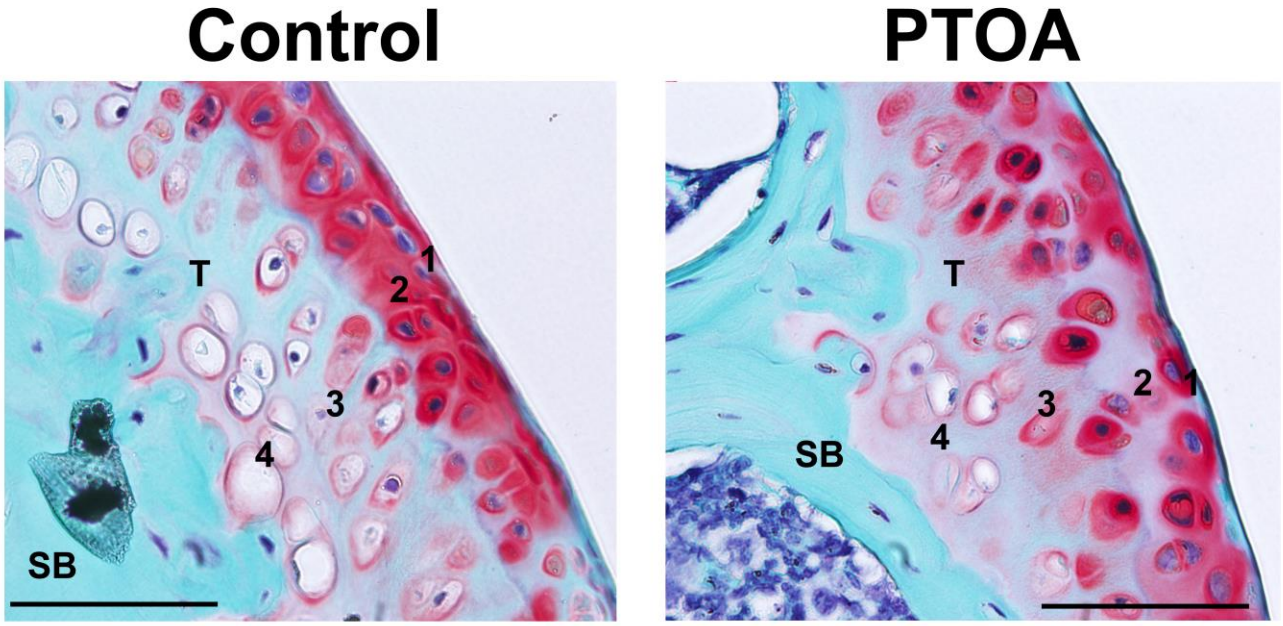

Figure 7. Comparison of Safranin-orange/fast green-stained four zones of the articular cartilage of the injured joint (right panel) versus the intact contra-lateral joint (left panel) five weeks post-surgery. 1, 2, 3, and 4 indicate zone 1, 2, 3, and 4, respectively. T indicates the "tidemark"; whereas SB is subchondral bone. The scale line represents 100 µm.

To assess the amount of cartilage loss in PTOA knee joints at the proximal tibial end at 5 weeks post-fracture, we measured the width (from the apex of the articular cartilage to the tidemark). The PTOA cartilage has lost almost 50% of its width (**Figure 9**), confirming substantial erosion of the articular cartilage in the PTOA joint.

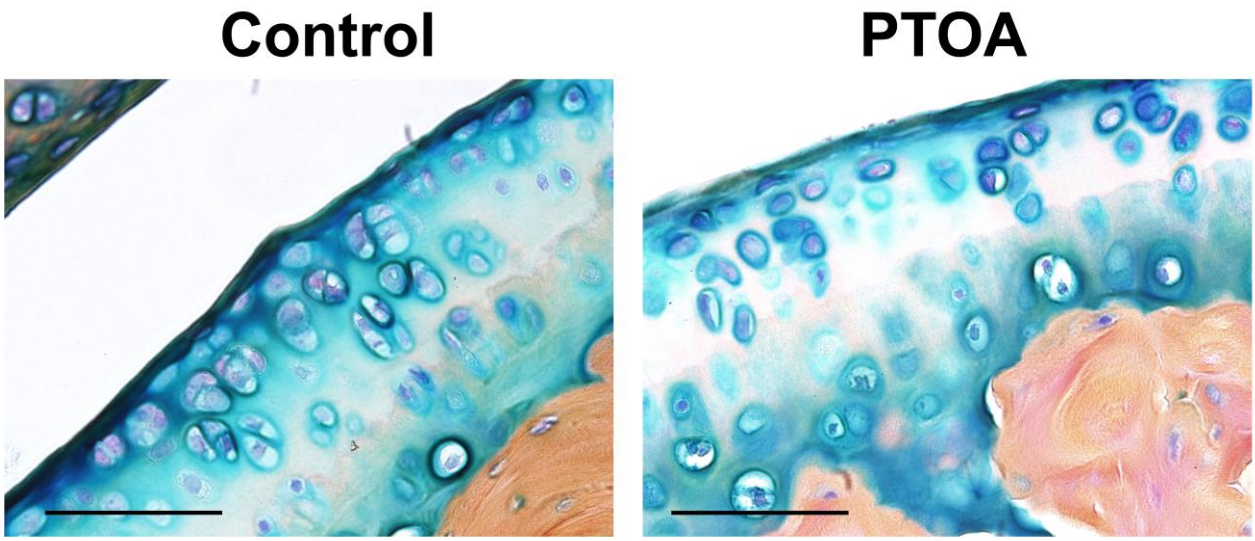

Figure 8. Comparison of Alcian blue/orange G-stained articular cartilage of the injured joint (right panel) versus the intact contra-lateral joint (left panel) five weeks post-surgery. The scale line represents 100 µm.

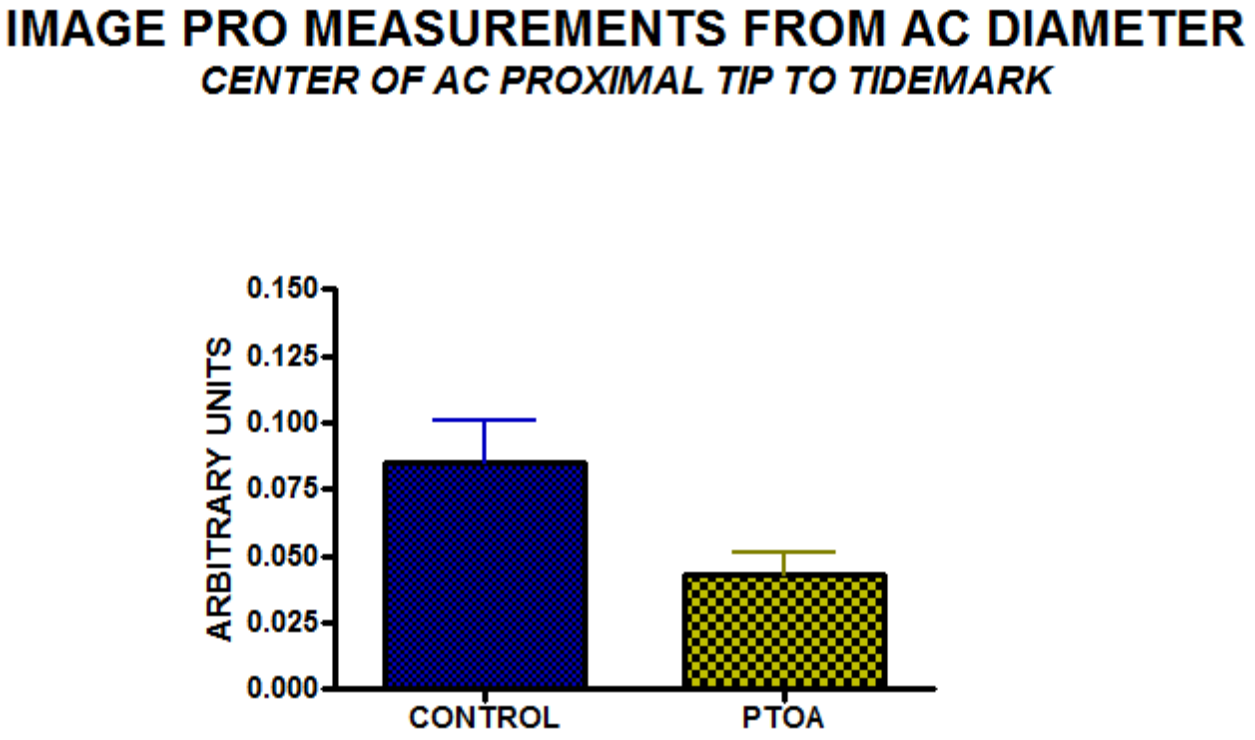

Figure 9. Comparison of the relative width (thickness) of the articular cartilage on PTOA right tibial end of 12-week-old mice at 5 weeks post-intra-articular tibial fracture with that of their contralateral intact left tibial. Relaitve distance was determined with an aid of the ImagePro software program.

On the basis of the foregoing histological characterizations, we have been exploring the possibility of deriving the following relative simple and straightforward semi-quantitative modified Minkin's OA scoring system to assess relative severity of PTOA. This method will base on four primary parameters: articular cartilage surface smoothless, loss of zones 1&2, extent of Safranin-O staining, and uniformity in rows of chondrocytes in Alcian blue-stained sections. This disease scoring system (**Figure 10**) will be tried in assessment of PTOA severe in our pending experiments of Tasks 3 & 4. Preliminary application of this PTOA scoring system to six PTOA knee joints yielded very high scores, whereas the contralateral intact knee joints gave a score of almost zero.

Score	0	1	2	3	4
Articular cartilage surface smoothless					
Loss of gliding and Intermediate zones	0%	10%	10-25%	25-50%	>50%
Safranin-O staining	No reduction	Slight reduction	Moderate reduction	Severe reduction	noted
Uniformity in Rows of chondrocytes	Normal	Reduced Hypercellularity	Clusters	Hypocellularity Defects to Cartilage	Disorganization Some rows absent Exposure to sub-chondral bone

Figure 10. A proposed semi-quantitative disease scoring system for assessment of relative severity of PTOA.

While these semi-quantitative disease scoring systems are useful, the histologic assessments of articular cartilage and subchondral bone destruction are of two-dimension and might not represent the complete picture of the disease. Consequently, these semi-quantitative methods frequently show large variations, which require large numbers of replicate for detection of small changes. More importantly, they can be substantially influenced by unintended biases. Accordingly, we set out to develop and validate a more direct three-dimensional quantitative measurement assay for PTOA during the reporting period.

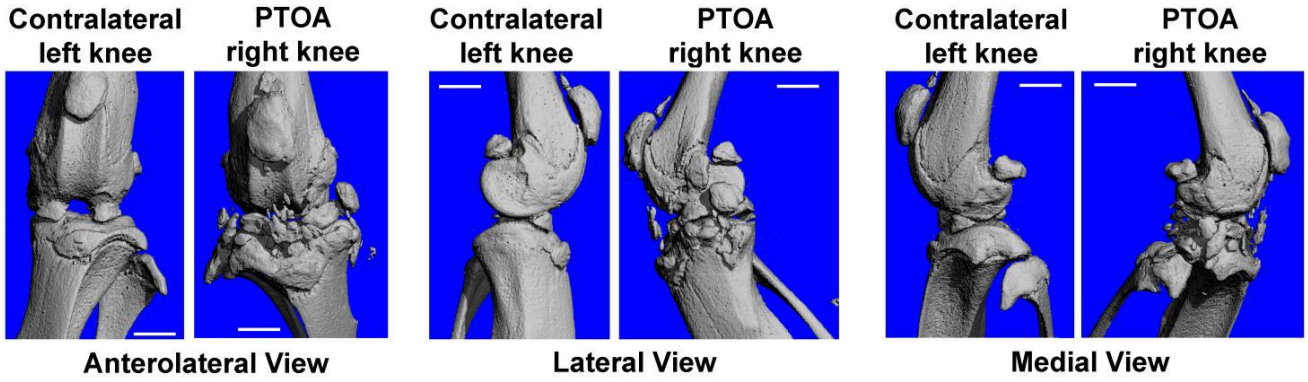

Figure 11. Three-dimensional micro-CT reconstruction images of injured knees 5 weeks post-fracture.

Osteophyte formation is a well-known characteristic of PTOA/OA. µ-CT technology is the state-of-the-art technology to measure three-dimensional parameters of calcified tissues, especially bone. Thus, µ-CT is an ideal technology to assess osteophyte in the three-dimensional context. The µ-CT reconstruction of the injured right knee joint and the contralateral intact left knee joints at anterolateral, lateral and medial views were then obtained (**Figure 11**). Significant numbers of mineralized bony tissues was found on the edges of the joint surface of PTOA joints but not on the joint surface of the uninjured contra-lateral control joints of the same mice at 5 weeks post-fracture, confirming that at five weeks after the intra-articular tibial plateau fracture insult, substantial amounts of osteophytes were found at and around the joint surface of the injured joint.

It is likely that before the injury the bone volume of the right knee at and around the articular cartilage surface was not different from that of the left knee. Thus, we can the assumption that the increase in bone volume (BV/TV) at and around the articular cartilage surface of the PTOA right knee compared to that of the contralateral left knee would be the bone volume of the osteophytes. Consequently, we sought to determine the relative amounts of osteophyte formation in PTOA by comparing the BV/TV of the injured knee joint with that of the contralateral uninjured knee joint at and around the articular cartilage surface by µ-CT. In this regard, we first chose four specific sites for measurement, i.e., from 0% (apex) to 100% distance between the apex of the articular surface and the growth plate of the tibia (the growth plate); from 0% (apex) to 30% distance between the apex and growth plate (approx. the edge of the articular cartilage surface); from 0% (apex) to 15% (the site of the tibial plateau where the fracture force was applied); and from 0% (apex) to 5% (where is the edge of the meniscus is located) (**Figure 12**).

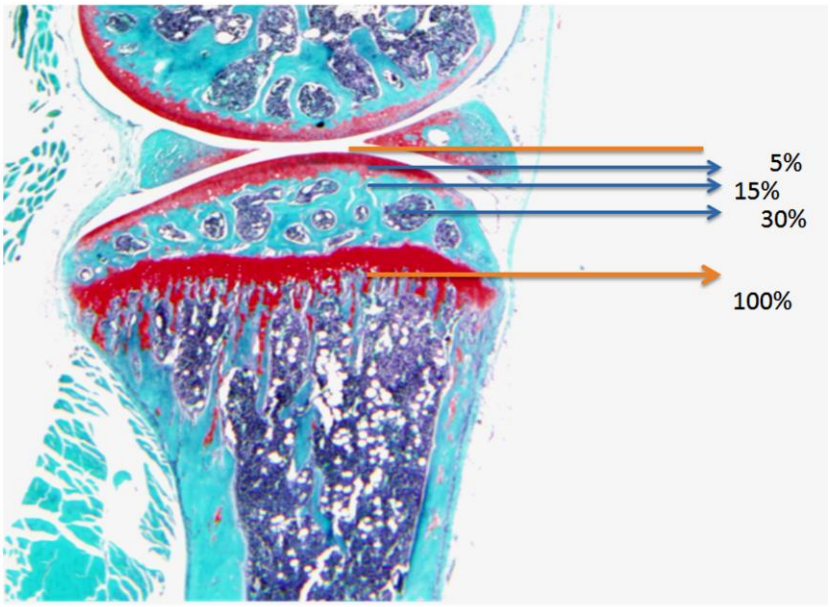

Figure 12. Sites of µ-CT measurement of bone volume as an estimation of osteophyte formation.

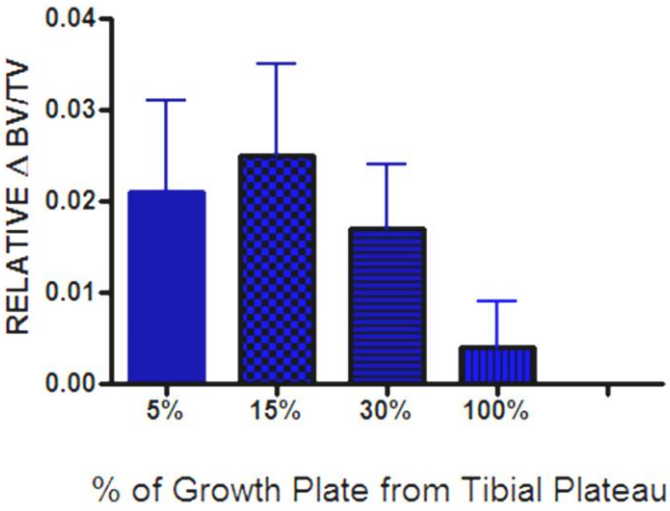

Figure 13. Relative differences in BV/TV (measured by µ-CT) at the selected distances from the growth plate between PTOA tibial plateau and contralateral uninjured tibial plateau of 12-week-old male C57BL/6J mice (n=6) at 5 weeks post-intra-articular tibial plateau fractures.

We have performed preliminary µ-CT measurements in groups of male and female 12-week-old C57BL/6J mice according to the foregoing scheme. We found significant increases in BV/TV in the right PTOA tibial plateau (compared to the left contralateral intact tibial plateau) at 5% (↑28.6±11.8%), 15% (↑28.9±12.5%), and 30% (↑28.6±12.2%) from the growth plate, but not at the growth plate (↑15.8±7.3%). These three-dimensional m easures are consistent with our two-dimensional measurements based on histology. Accordingly, Safranin O measurements also showed 20.8% increase in osteophyte area compared with the articular cartilage measurement following the tidemark to the apex of the AC using osteomeasure software (**Figure 14**). Similar results were obtained with 12-week-old female mice (data not shown).

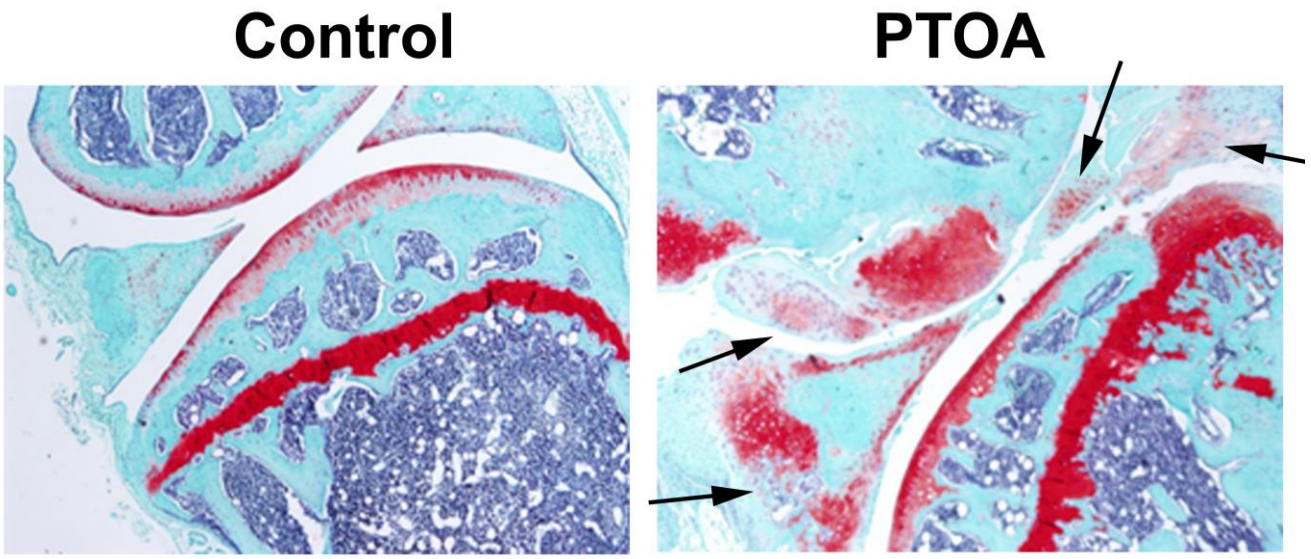

Figure 14. Comparison of osteophyte areas (based on Safranin-O staining measurements of extraskeletal bony tissues between the apex of the articular cartilage to the tidemark using an osteomeasure software) between PTOA tibial plateau and contra-lateral uninjured tibial plateau of 12-week-old male C57BL/6J mice at 5 weeks post-fracture. Arrows indicate extraskeletal bony tissues.

We are currently attempting to validate this µ-CT-based measurement of osteophyte area method as an index of the severity of PTOA by correlating between the increases in BV/TV at the articular cartilage surface with the histologically-based disease score as described above. A strong correlation would support the validity of this method.

We also made another intriguing observation in that the chondrocytes within the meniscus of the PTOA joint at 5 weeks after the intra-articular fracture became highly hypercellular when compared to the meniscus of the contralateral uninjured knee (**Figure 15**). Meniscus is made of a different type of cartilage (fibroelastic cartilage) than the articular cartilage (hyaline cartilage). Accordingly, it seems that cellular factors that are responsible for the increased cellularity in the hyaline cartilage may also induce hypercellularity in chondrocytes of the fibroelastic cartilage.

Accordingly, we are also interested in the possibility that measurements of the chondrocyte hypercellularity in the meniscus of the PTOA joint could be a useful surrogate index of the severity of PTOA. To explore this possibility, we measure the hypertrophic chondrocyte and total tissue area of the meniscus of both the PTOA and contralateral intact control joints (left panel of **Figure 16**). We found that there was relatively small portion of tissue area (<10%) of the meniscus of the intact knee joint that was occupied by hypertrophic chondrocytes. Conversely, more than 50% of the total meniscus area of the PTOA joint was filled with hypertrophic chondrocytes (right panel of **Figure 16**). We are currently testing the possibility of whether the relative percentage of hypertrophic chondrocyte area in PTOA could be a valid index of relative severity of the PTOA.

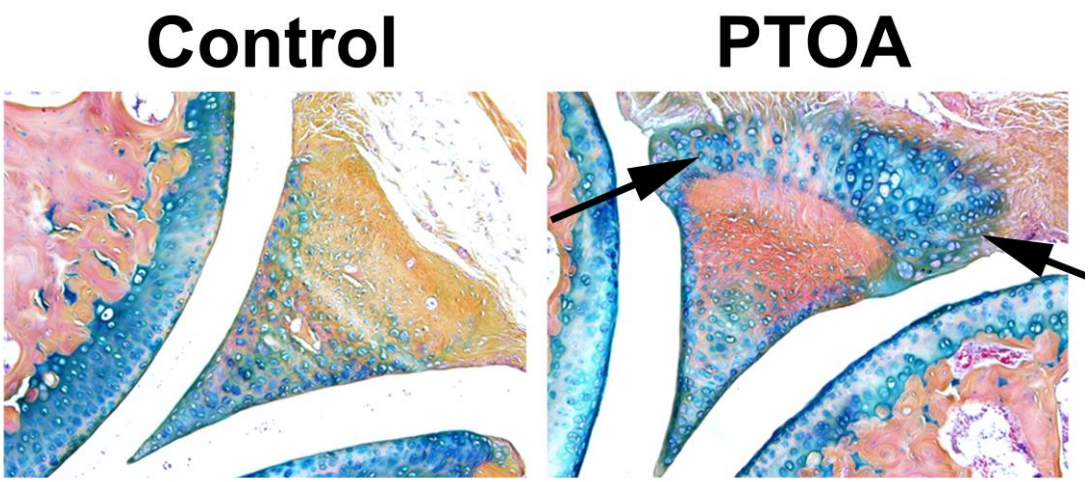

Figure 15. Injury in the intra-articular tibial plateau increased chondrocyte hypercellularity of the fibroelastic cartilage of the meniscus. Longitudinal thin sections (5 µm) of the femoral-tibial junction, including the meniscus, were stained with alcian blue with orange G counterstain. GAG-proteoglycan of cartilage is stained in blue/purple color, and bone is stained in orange to red color.

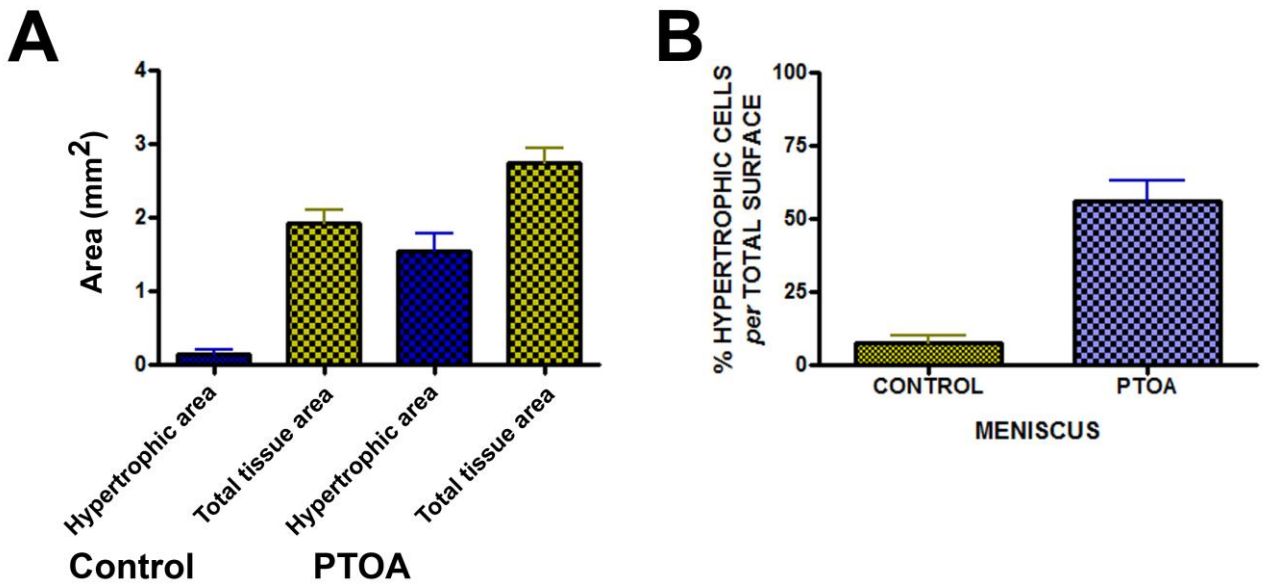

Figure 16. The hypertrophic chondrocyte and total tissue areas of the meniscus of both the injured right knee joint as well as the control contralateral intact knee joint were determined with the aid of an osteomeasure software (panel A) and the comparison of the relative ratio of the hypertrophic chondrocyte area to total tissue area of meniscus of the PTOA joints with that of contralateral control joint (panel B).

Before we begin to develop and evaluate the efficacy of the EfnA4-fc chimeric protein-based therapy for PTOA, we initiated the second phase of the subtask, which is to determine how long the injected soluble EfnA4-fc chimeric protein would remain in the synovium after injection. For this work, we injected 4 mice with ~ 2-5 µl of 1% aqueous crystal violet solution into both legs supra laterally or from the side of the patella into synovial space. At day 1 and day 4 after the dye injection, two mice were sacrificed at each time point, and the limbs containing the femur and the tibia as well as the knee joint were dissected out.

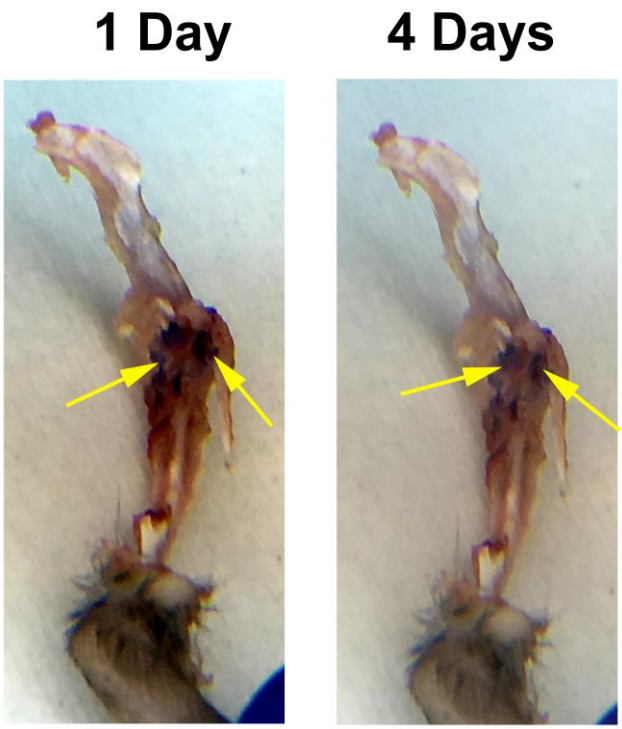

Figure 17. Location and retention of the injected violet crystal solution at the injected knee joint at 1 day or 4 days post-injection. Yellow arrows point to the location of the retained violet crystal at the knee joint.

As shown in **Figure 17**, substantial amounts of the inject crystal violet dye was seen after 1 or 4 days post-injection. On day 1, a lot of crystal violet dye was found in the bone marrow and muscle as well as in the area near the lateral patellar joint. Upon dissection of the joint sagitally, the area was in close proximity to the synovium (not shown). On day 4 after crystal violet injection, the dye was shown to diffuse into the immediate muscle area. However, upon dissection, there was no crystal violet present (not shown), suggesting that the dye had already migrated into the muscle and out of the synovium at this time point. If we assumed the soluble EfnA4-fc chimeric protein migrates as freely as soluble dyes, such as crystal violet in the synovium of the knee joint, this preliminary finding suggest that the injected protein should stay around at the injection site for approximately 4 days. This information would be important and relevant to our design of our EfnA4-fc chimeric protein injection procedure. Specifically, it would suggest that the clearance rate of the injected protein would be less than 4 days. Thus, multiple injections may be required for the optimal therapeutic effect. However, we are currently testing if the use of an appropriate hydrogel as the delivery scaffold prolongs the biological availability of the injected soluble protein in the PTOA synovial. We have tried the collagen-based collagen-based hydrogel scaffold and fold that the diffusion of dye out of the synovium can be extended to at least one week. However, since collagen can induce inflammation at the synovium and the joint, we will no longer consider collagen-based hydrogels for our work. One of the hydrogels that we are considering is the hyaluronic acid-based hydrogel, since hyaluronic acid is a key component of articular cartilage and has been suggested to have protective effects on arthritis.

In summary, subtask 3.1 is ~15% completion.

iii. *Subtask 4.1: To determine whether injection of a high dosage of EfnA4-fc every other week into injured knee joints could prevent PTOA development*.
We have just initiated a preliminary study to evaluate whether injection of a soluble EphA4-binding ephrin (i.e., EfnA4-fc) every other week into injured knee joint of 12-week-old mice for five weeks would reduce or

prevent development of PTOA. Very briefly, the right knee of six 12-week-old C57BL/6J mice was subjected to intra-articular tibial plateau fracture with a 55-N force using an Instron mechanical tester. A dose of 2 μg/kg EfnA4-fc (without hydrol) in PBS was directly injected into the injured synovium 24 hrs later and every week for four weeks thereafter. EfnA4-fc was used in this experiment because it was effective in stimulating the forward signaling of EphA4 in vitro. The chosen dosage was based on an effective dose used by another laboratory to activate the EphA4 signaling in the brain. In this preliminary study, we did not include a un-injected control group, since we plan to compare our results with those we have already gotten from the current preliminary studies. If the study is encouraging, we will repeat this study with appropriate control groups. The mice will be euthanized after eight weeks in October. We will then determine the severity of PTOA in the injured joints using the methods that are being developed in our laboratory.

Secondly, while we tried to quantitate the extent of the post-traumatic osteoarthritis, we found that none of the methods reported in the literature was reliable, because they are very subjective and based primarily on histology. Bone histology is a good qualitative proof for development of PTOA, but it is not a reliable quantitative method, since it is so subjective to the site of examination, which is highly variable and also is subjective to confounding factors due to variation in morphology and bone and cartilage architecture. We reason that since we want to prove that our therapy can reduce or prevent development of PTOA, it is essential that we need to have a reliable quantitative method. Otherwise, our interpretation may be subjected to unintended biases due to inappropriate selection of sites of examination.

One of the potential means for a good quantitative assessment of the severity of PTOA would be the micro-CT measurement of osteophyte. [Micro-CT is a great technology to assess bone content in a three dimensional way}. Therefore, I thought it would be a good investment of our time to evaluate whether this approach would be useful.

In summary, subtask 4.1 is only ~5% completion.

4. Brief Summary of Key Accomplishments:
    - We have successfully demonstrated that activation of the forward signaling of EphA4 in synoviocytes suppressed the release of pro-inflammatory cytokines and degradative proteases, such as MMPs in vitro (03-15-2016).
    - We have successfully demonstrated that deficient in expression of EphA4 in the synovium significantly upregulated the expression of pro-inflammatory cytokines and MMPs, but without an effect on the expression of EphA4-binding ligands or articular cartilage marker genes (06-15-2016).
    - We have successfully demonstrated that deficient in expression of EphA4 in the articular cartilage significantly suppressed the expression of marker genes of articular cartilage maturation and differentiation (06-15-2016).
    - We have demonstrated that application of a 55-N force at the intra-articular tibial plateau, which was sufficient to create injury at the joint but not sufficient to create fracture, consistently and reproducibly caused the development of PTOA after 4 weeks (03-15-2016).
    - We have shown that μ-CT can be used to quantitative assess the volume of osteophytes produced at or around the articular cartilage surface, which may be developed into a quantitative assay method for the severity of PTOA/OA (09-15-2016).
    - We have shown that PTOA development also caused hypercellularity of the chondrocytes in to fibroelastic cartilage of the meniscus, which may also be used as a quantitative method to assess the severity of PTOA/OA (09-15-2016).
    - We have shown that direct injection of a soluble material, such as EfnA4-fc, with scaffold, can stay inside the synovium for 4 to 7 days (06-15-2016).

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

- Complete Task 2 by developing and optimization of EfnA-fc-based therapy for PTOA.
- Initate work of Task 3 to establish therapeutic efficacy of the EfnA-fc-based therapy in prevention and treatment of PTOA.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

There are currently no convenient and validated quantitative methods to assess the relative severity of PTOA/OA in an animal model. If our current effort focusing on development of such a method is successful, our successful establishment of such methods would undoubtedly facilitate and speed up the progress of current and future development of effective therapeutic agents to treat PTOA/OA. Therefore, our work during the reporting period in this regard has important impact.

What was the impact on other disciplines?

Nothing to report at this time.

What was the impact on technology transfer?

Nothing to report at this time.

What was the impact on society beyond science and technology?

Nothing to report at this time.

5. CHANGES/PROBLEMS:

Our findings that synoviocytes (and perhaps also articular chondrocytes) express multiple EphA4-binding ephrin's suggest that our original strategy to use the soluble EphA4-fc chimeric protein to suppress the forward signaling of EphA4 in the synovium and on articular chondrocytes would be highly complicated and not likely yield definitive results. Our preliminary use of isolated synoviocytes from EphA4 null mice indicates that the use of EphA4 null mice would be a better and more attractive approach. Consequently, we plan to submit an amendment to our local IACUC requesting the use of EphA4 null mice in this project. Once it is approved, we will submit an amendment to ACURO for approval. Once approval is obtained by both our local IACUC and ACURO, we will immediately submit to DoD for permission to amend our Technical Objectives to also include the use of EphA4 null mice.

Actual or anticipated problems or delays and actions or plans to resolve them
them.

The Instron Mechanical Tester, used by use to create reproducible intra-articular tibial plateau fractures, was broken down during the early months of this reporting period. This has led to a significant delay in our progress towards Tasks 2 and 3. Fortunately, this issue has been satisfactorily resolved in May 2016. We do not foresee any similar issues in the future.

Changes that had a significant impact on expenditures

We do not anticipate our proposed changes in using the EphA4 null mice would have any significant impact on the overall expenditures of this project.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

There were no significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of vertebrate animals

There were no changes in use or care of vertebrate animals. However, as indicated in the detailed progress report section, we plan to use EphA4 null mice to evaluate the effect of the lack of EphA4 signaling in the articular cartilage and infiltrating synovial macrophages on the development of PTOA. We will submit amendment to our IACUC for approval. Once we secure approval for the amendment, we will submit an abbreviated ACURO application for approval of the amended use of EphA4 null mice for this project.

Significant changes in use of biohazards and/or select agents

There were no significant changes in use of biohazards and/or select agents.

6. PRODUCTS:

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications.

  Nothing to report at this time.

  Books or other non-periodical, one-time publications.

Nothing to report at this time.

Other publications, conference papers and presentations.

Nothing to report at this time.

- **Website(s) or other Internet site(s)**

Nothing to Report.

- **Technologies or techniques**

Nothing to report at this time.

- **Inventions, patent applications, and/or licenses**

Nothing to report at this time.

- **Other Products**

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Kin-Hing William Lau, Ph.D.*
Project Role: *Principal Investigator*
Researcher Identifier (e.g. ORCID ID): 0000-0003-1109-5052
Nearest person month worked: *3.6*

Dr. Lau is the Principal Investigator and is in charge of all aspects of this project.

Funding Support: *VA BLR&D Merit Review – Dr. Lau's salary support is provided by a VA BLR&D Research Career Scientist Award.*

Name: *Virginia Stiffel, B.S.*
Project Role: *Senior Research Technician*
Researcher Identifier (e.g. ORCID ID): None
Nearest person month worked: *12*

Ms. Stiffel is a senior Research Associate, who has worked with and trained by Dr. Lau for more than 15 years. She is responsible for daily operation and execution of the project. She also assists Dr. Lau in training and supervising Ms. Das, the Research Technician of this project.

Funding Support: *Ms. Stiffel's salary support comes 100% from this DOD project.*

Name: *Subhashri Das, B.S.*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID): None
Nearest person month worked: *12*

Ms. Das is responsible for daily execution of the project.

Funding Support: Ms. Das's salary support comes 100% from this DOD project.

Name: *Matilda Sheng, Ph.D.*
Project Role: *Co-Investigator*
Researcher Identifier (e.g. ORCID ID): 0000-0002-0815-7008
Nearest person month worked: *1.2*

Dr. Sheng is responsible for supervising Research Technicians in carrying out histological analyses of PTOA samples.

Funding Support: Dr. Sheng's salary support comes from Loma Linda University.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Lau, the Principal Investigator has recently granted a 4-year VA BLR&D Merit Review grant to investigate the regulation and functional role of miRNA-17 in controlling the functional activity of mature osteoclasts. There are no scientific, administrative, or financial overlaps of this Merit Review project with the current DoD project. There is no change in percent efforts of any key personnel spending on this project. Thus, no change in financial or scientific aspects of this project is requested.

What other organizations were involved as partners? N/A

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES: N/A